Role of gap junctions during early embryo development

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Abstract

Gap junctional communication plays a central role in the maintenance of cellular homeostasis by allowing the passage of small molecules between adjacent cells. Gap junctions are composed of a family of proteins termed connexins. During preimplantation development several connexin proteins are expressed and assembled into gap junctions in the plasma membrane at compaction but the functional significance of connexin diversity remains controversial. Although, many of the connexin genes have been disrupted using homologous recombination in embryonic stem cells to obtain unique phenotypes, none of these studies has demonstrated a specific role for connexins during preimplantation development in the null mutants. This review surveys evidence for the involvement of gap junctional communication during embryo development highlighting discrepancies in the literature. Although some evidence suggests that gap junctions may be dispensable during preimplantation development this is difficult to envisage particularly for the process of cavitation and the maintenance of homeostasis between the differentiated trophectoderm cells and the pluripotent inner cell mass cells of the blastocyst.


Gap-junction structure

Gap junctions maintain cellular homeostasis by allowing communication between adjacent cells. They span the plasma membrane of two adjacent cells (Makowski et al. 1977), with each cell contributing half the channel; a hemichannel, or connexon. A connexon from one cell docks in the extracellular space with a connexon from an opposing cell to form a complete gap-junction channel, allowing adjacent cells to be coupled (reviewed by Bruzzone et al. 1996a). Each connexon is a multimeric assembly of six proteins, termed connexins (Fig. 1). Connexons may be composed of the same type of connexin, termed homomeric, or contain multiple connexins, termed heteromeric connexons. The docking of two homomeric connexons composed of the same connexin protein yields a homotypic channel, whereas the oligomerization of two homomeric connexins composed of different connexins forms a heterotypic channel (Revel & Karnovsky 1967, Makowski et al. 1977, Unwin & Zampighi 1980, Casio et al. 1995). Studies using atomic force microscopy have shown that liver gap junctions are densely packed with a centre-to-centre distance of 9–10 nm and an average pore size of 3.9 nm (Hoh et al. 1993, Lal et al. 1995). Each connexon forms a cylinder with a pore in the centre through which molecules of less than 1000 Da, such as metabolites, ions and second messengers, can pass, thus facilitating homeostasis (reviewed by Bruzzone et al. 1996a).

Currently 19 rodent and 20 human connexins have been characterized (Willecke et al. 2002). They vary in both the properties of the channels they form as well as their distribution among adult cell types, with most cells expressing more than one connexin type. Connexins are integral membrane proteins containing four transmembrane, two extracellular and three intracellular domains. The intracellular domains, which contain both the C- and N-terminal segments and intracellular loop, are targets for post-translational modifications, important for the regulation of channel activity. The extracellular domains are required for connexon docking. Connexins are classified according to their molecular mass (Beyer et al. 1987). For example, connexin (Cx)43 has a predicted molecular mass of 43 kDa and Cx31 has a predicted molecular mass of 31 kDa. Co-expression of connexins is common; hepatocytes express both Cx26 and Cx32 (Nicholson et al. 1987), as do proximal kidney tubule cells (Butterweck et al. 1994), whereas vascular endothelium co-expresses Cx37 and Cx40 (Delorme et al. 1997). Gap junctions composed of different connexins have different rates of diffusion for specific molecules (reviewed by Koval 2002). For example, Cx43 channels are 120–160-fold more permeable to ADP and/or ATP than Cx32 channels (Goldberg et al. 1999). In addition, the formation of heterotypic gap junctions enables unique permeability characteristics to be obtained compared to homotypic channels. Channels composed of Cx32 allow the passage of cGMP and cAMP whereas heteromeric
Cx32 and Cx26 channels are preferentially permeable to cGMP compared to cAMP (Bevans et al. 1998); however, the mechanism of action has yet to be determined.

Connexin expression in preimplantation embryos
Connexin mRNA expression patterns vary during murine preimplantation development with Cx30, Cx31, Cx36, Cx43, Cx45 and Cx57 being expressed from the two- to four-cell stage, and Cx30.3, Cx31.1 and Cx40 from the eight-cell stage (Davies et al. 1996, Houghton et al. 2002). Connexin proteins tend to be translated shortly after mRNA expression but are located initially in the cytoplasm. Once cell adhesion between blastomeres has occurred at the eight-cell stage, in the process of compaction (reviewed by Fleming et al. 2001), Cx43 begins to traffic to the plasma membrane for assembly into gap junctions (De Sousa et al. 1993). It has been shown that although compaction and gap-junction formation in embryos are independent events, they are temporally correlated (Kidder et al. 1987). Appreciation of the involvement of cell-adhesion molecules in gap-junction formation arises from work using a mouse sarcoma cell line (S180). These cells express Cx43 but fail to display coupling unless they are transfected with E-cadherin, a calcium-dependent cell-adhesion molecule (Mege et al. 1988) that is also observed in other cell types (Jongen et al. 1991). It is likely that the control of gap-junctional intercellular communication by E-cadherin involves post-translational regulation (assembly and/or function) of the gap-junction protein Cx43 (Musil et al. 1990).

Human preimplantation embryos express predominantly Cx43 and protein levels increase throughout development to the blastocyst stage (Hardy et al. 1996). This finding was confirmed and extended at the mRNA level where Cx31 and Cx43 were found to be expressed throughout development; Cx26 and Cx45 showed inconsistent expression, whereas Cx32 and Cx40 were not expressed at any stage (Bloor et al. 2004). This is in contrast to Hardy et al. (1996), who found the presence of Cx32 protein in the late human blastocyst. The functional significance of the inconsistently expressed connexin genes has yet to be elucidated. At the blastocyst stage, Cx26, Cx45 and Cx31 showed a reduced level of protein
expression compared to Cx43, but displayed coexpression with Cx43 (Bloor et al. 2004).

In the bovine embryo, Cx43 expression varies depending on whether the embryos are produced in vitro or in vivo. In vitro, Cx43 was expressed in the oocyte and zygote through to the morula stage but was not expressed at the blastocyst stage, whereas Cx43 transcripts were detected in morula and blastocysts produced in vivo (Wrenzycki et al. 1996). Subsequently, a significant increase in Cx43 mRNA expression was found from the 16-cell stage to the blastocyst stage during in vivo bovine development (Lonergan et al. 2003).

**Signalling and gap-junction regulation**

Gap junctions may be regulated by hormones and other extracellular signalling molecules such as neurotransmitters, growth factors and cytokines (Stagg & Fletcher 1990, Bruzzone et al. 1996b, Sáez et al. 1998). The extent to which these processes are coupled depends on several mechanisms: gene transcription, stability of the message, translational and post-translational modifications and assembly of the protein into the membrane (Sáez et al. 1998). Connexin phosphorylation occurs predominantly in the C-terminal domain and is the best-characterized mechanism of regulation, mediated predominantly by protein kinase A (PKA), protein kinase C (PKC), mitogen-activated protein kinases and tyrosine kinase (reviewed by Sáez et al. 1998, Lampe & Lau 2004). Depending on the cell type, these kinases may either increase or decrease gap-junctional intercellular communication (reviewed by Alves et al. 2000). For example, in bovine lens cells, activation of PKC inhibits gap-junctional communication (Reynhout et al. 1992) whereas in neonatal rat cardiomyocytes activation of PKC increases junctional conductance (Kwak et al. 1995). In the preimplantation embryo, Cx43 is present in the non-phosphorylated form from the mid-four-cell stage with the phosphorylated form increasing from the eight-cell stage onwards (Ogawa et al. 2000). These investigators also found that activation of PKA via dibutyryl-cAMP increased Cx43 phosphorylation and the number of Cx43-positive plaques, whereas a PKC activator, tumour promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) increased Cx43 phosphorylation but decreased the number of Cx43-positive plaques. Taken together, these results suggest that phosphorylation is required for gap-junction formation in the embryo and may be facilitated by PKA and inhibited by PKC activation.

**Functional significance of connexin diversity**

Although each connexin possesses distinct gating properties and its conductance may be controlled by factors such as Ca$^{2+}$, pH and cAMP, their functional significance during preimplantation development is unknown. In an attempt to explore the functional significance of connexin diversity, a targeted gene-inactivation approach has been used. Several of the murine connexin-encoding genes have now been disrupted using homologous recombination in embryonic stem cells: Cx43 (Reaume et al. 1995), Cx32 (Nelles et al. 1996), Cx37 (Simon et al. 1997), Cx46 (Gong et al. 1997), Cx40 (Simon et al. 1998), Cx26 (Gabriel et al. 1998), Cx50 (White et al. 1998), Cx45 (Kumai et al. 2000), Cx36 (Guldenagel et al. 2001), Cx31 (Plum et al. 2001) and Cx30 (Teubner et al. 2003). Each connexin-null mutant results in a unique phenotype, demonstrating a specific physiological role for that connexin in at least a subset of its expression sites (reviewed by Nicholson & Bruzzone 1997, White & Paul 1999). However, these phenotypes are often difficult to interpret due to the potential for redundancy and for other co-expressed connexins to compensate for loss of expression. For example, Cx43 is expressed in the preimplantation embryo from the two-cell stage onward (De Sousa et al. 1993). Nevertheless, mice lacking Cx43 survive to term, but die shortly afterwards due to a morphological defect in the right-ventricular outflow tract of the heart (Reaume et al. 1995). However, the lack of abnormalities in other tissues is often used as evidence that compensation occurs between connexins, or that there is redundant expression.

The possibility that connexins may functionally compensate for one another in cells where they are co-expressed was studied by generating doubly mutant mice, deficient in Cx32 and Cx43. Fetuses lacking both Cx43 and Cx32 survived to term but died shortly afterwards from the same cardiac abnormality associated with the Cx43 deficiency. No morphological abnormalities were observed in the limbs, thyroid gland or developing teeth, the major sites where the two connexins are co-expressed (Houghton et al. 1999).

**Gap junctions and preimplantation development**

Whether or not there is a functional requirement for gap junctions in preimplantation development is controversial. Becker et al. (1995) raised several anti-Cx43 antibodies to different regions of the protein to test their ability to perturb gap-junctional intercellular communication in mouse embryos. It was found that injection of anti-peptide antibodies designed to the cytoplasmic loop of Cx43, together with the dyes Lucifer Yellow or Cascade Blue, was effective in blocking the transfer of dye between blastomeres of 8–16-cell embryos. In addition, the cell containing the blocking antibody decompacted and was excluded from further development (Becker et al. 1995), suggesting that good gap-junctional communication is essential for compaction and embryo development (Becker & Davies 1995). These experiments may be interpreted in light of the results obtained from Cx43-null homozygous mutant embryos, which develop normally and establish full-term pregnancies (Reaume et al. 1995). Embryos lacking Cx43 have been shown to display a severely reduced level of dye coupling with altered permeability characteristics (De Sousa et al. 1997). Thus, Cx43-null homozygous morulae
were found to be uncoupled when injected with 6-carboxyfluorescein, but when 2',7'-dichlorofluorescein was used coupling was evident. These permeability characteristics are typical of Cx45 channels, although the possible involvement of other connexins cannot be ruled out. Gap-junction channels are differentially and selectively permeable to various dyes (Elfgang et al. 1995). This may explain the apparent lack of coupling in embryos injected with anti-Cx43 antibodies using an anionic dye like Lucifer Yellow, which is transmitted poorly in Cx45 channels (Steinberg et al. 1994).

The importance of gap-junctional communication has been studied using the inhibitor 18α-glycyrrhetinic acid (AGA), which completely abolished dye coupling in preimplantation embryos without affecting blastocyst formation, or cell allocation to the trophectoderm or inner cell mass (Vance & Wiley 1999). These results were surprising but suggested that gap-junctional intercellular communication was not required for successful development of the preimplantation embryo. However, it is possible that coupling-deficient embryos display secondary, cellular or metabolic defects since it is known that signalling molecules may be transmitted via gap junctions with implications in cell protection via programmed cell death (Bannerman et al. 2000). However, there was no observable difference in TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling) staining between AGA-treated and control blastocysts (Houghton et al. 2002). Transport through gap junctions has also been identified as a potential rate-limiting factor in glucose utilization by cultured cells (Giaume et al. 1997). Again this seems not to be the case in the preimplantation embryo since there was no significant difference in glucose and pyruvate consumption and lactate production in blastocysts cultured in the presence or absence of AGA (Houghton et al. 2002). Taken together, these results suggest that gap-junctional intercellular communication is not obligatory for preimplantation development. However, this conclusion should be treated with caution since relatively few reagents are known to block gap-junctional intercellular communication in a specific manner. AGA indirectly blocks gap junctions through activation of protein kinases, G-proteins or transport ATPases (Evans & Boitano 2001). This causes changes in the phosphorylation of the connexin C-terminal tail, particularly Cx43 and Cx45, affecting channel gating and assembly into functional gap junctions (Evans & Boitano 2001, Zucker & Nicholson 2002). Nine connexins are expressed during murine preimplantation development and hence it is difficult to know whether intercellular communication is dispensable, since there are currently no specific inhibitors capable of blocking all the potential types of channel. In addition, although gap junctions may appear dispensable to preimplantation development, the ability of uncoupled blastocysts to produce viable offspring following embryo transfer has yet to be tested.


In summary, controversy still surrounds the functional requirement of gap junctional intercellular communication in the preimplantation embryo. If gap junctions are not obligatory, at least in the mouse, it is legitimate to ask: whether they have some as-yet undiscovered role, perhaps in vivo; whether they are expressed precociously in anticipation of some future event(s); and whether they provide a fail-safe function for contingencies that might arise in vivo.

In response to the first question, the major feature of development in vivo, absent in vitro, is obviously the presence of the maternal compartment. It is likely that embryo–maternal signalling will occur during preimplantation development and that maternally derived molecules will need to be distributed rapidly and evenly between the cells of the early embryo to ensure a consistent response. This homeostatic mechanism would be facilitated by the presence of gap junctions and may contribute to the superiority of in vivo over in vitro development in terms of blastocyst formation rate and cell number (Bowman & McLaren 1970).

Regarding the second question, it has been proposed that multiple connexins are expressed in the preimplantation embryo to ensure their coordinated and rapid segregation at implantation (Houghton et al. 2002). For example, Cx31 and Cx43 are expressed abundantly in both cell lineages of the blastocyst, the inner cell mass and trophectoderm, but upon implantation Cx31 is restricted to the ectoplacental cone and extraembryonic ectoderm, while Cx43 is found in the embryo and visceral endoderm (Dahl et al. 1996, Grümmer et al. 1996).

Finally, it is possible that gap junctions are required for the early embryo to respond with maximal efficiency to stresses encountered in vivo, for example, by delayed fertilization, prolonged transit through the Fallopian tube or early entry into the uterus.

Conclusion
It is difficult to envisage that gap-junctional intercellular communication is not required during preimplantation development, at least for blastocyst formation. The formation of gap junctions during compaction will ensure their presence at cavitation and in the trophectoderm and inner cell mass. The trophectoderm is the first epithelium
and, by analogy with adult epithelial tissues, acts as a selective entry and exit system to a variety of molecules ultimately required by the cells of the inner cell mass (Brison et al. 1993, Hewitson & Leese 1993). It is essential that the trophectoderm acts as a functional unit, a role that will be facilitated by the presence of gap junctions to ensure consistency in response to extracellular signals or those derived from the inner cell mass via the blastocoeal fluid.

Acknowledgements

I would like to thank Professor Henry Leese for his critical reading of the manuscript and many helpful discussions. F D H is supported by a Wellcome Trust Research Career Development Fellowship. The author declares that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received 15 April 2004
First decision 18 May 2004
Revised manuscript received 16 November 2004
Accepted 22 November 2004